



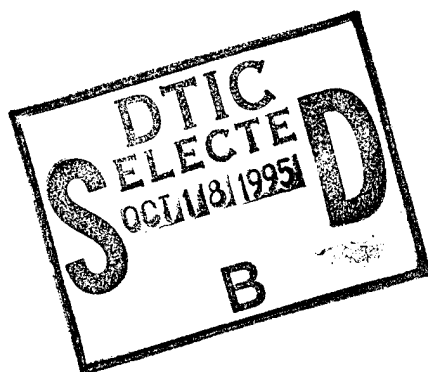
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U.S. ARMY CHEMICAL AND BIOLOGICAL DEFENSE COMMAND

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**SENSITIVITY INCREASE
IN NICOTINIC ACETYLCHOLINE RECEPTOR BINDING
IN FIBER OPTIC WAVE GUIDE**



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EXECUTIVE SUMMARY

Ligand binding studies with nicotinic acetylcholine receptor on the fiber optic biosensor indicate that while K_i values for antagonists were similar to those obtained in membrane binding assays, the agonists appear much less potent. This may be due to the inability of the receptor to make appropriate conformational changes necessary for agonist binding. Further studies on the Light Addressable Potentiometric Sensor, in which receptor proteins are immobilized using antibodies as spacer molecules, have shown greater agonist binding affinity than obtained on the fiber optic biosensor. To test whether spacer arms increase the conformational freedom of receptor protein immobilized on fibers, the nAChR was tethered to the fiber using an anti-nAChR antibody. In our present studies, rat anti-nAChR antibodies were immobilized onto the quartz fibers. The antibody-loaded fibers were then incubated in purified nAChR, and probed with a fluorescein-labeled ligand. The K_i values for antagonists were similar to those from previous studies, while the K_i values for acetylcholine and carbamylcholine were at least one order of magnitude lower than previously reported. With the exception of alpha-naja toxin, the values derived from the potentiometric sensor were two orders of magnitude lower than values obtained from the fiber optic biosensor.

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SENSITIVITY INCREASE IN NICOTINIC ACETYLCHOLINE RECEPTOR BINDING ON FIBER OPTIC WAVE GUIDE

1. INTRODUCTION

Biosensors are analytical devices that quantitatively detect the presence of specific chemicals, toxins, or organisms in various media. The biological binding events of biomolecules immobilized onto transductive devices are converted into electrical or optical signals. The fiber optic evanescent wave sensor has been demonstrated to be sensitive in detection using neuroreceptors, antibodies and enzymes⁽¹⁻³⁾. This sensor detects molecules within the evanescent wave zone which extends only a fraction of a wavelength above the surface of the fiber. Binding events on the surface of the fiber are easily monitored without interference from the bulk solution. Excitation of fluorophores occurs when some of the light transmitted down the fiber enters the evanescent zone. Fluorophore emission at a higher wavelength is trapped in the fiber and is transmitted back through the fiber to a detector.

With the establishment of proof of principle of biosensors comes the necessity of refinement in the areas of size, specificity, stability, and sensitivity. Many chemical vapor sensors are available as handheld instruments, but the downsizing of biosensors remains future. Requisite specificity in antibody-based biosensors is provided by monoclonal antibodies, while neuroreceptors are useful for more generic sensing within a class of compounds. The stability of biomolecules has been increased by covalent immobilization involving silanes and crosslinkers, use of polyphenylurethane linkages, and by drying in solutions of trehalose^(4,6). Biosensor sensitivity has been improved by the development of the Light Addressable Potentiometric Sensor (LAPS), the surface plasmon resonance instrument, and the use of tapered fibers in laser-driven waveguides⁽⁷⁻⁹⁾.

Previous ligand binding studies with nicotinic acetylcholine receptor (nAChR) on the fiber optic sensor indicated that while K_i values for antagonists were similar to those obtained in membrane binding assays, the agonists appeared much less potent. This may be due to the inability of the directly immobilized receptor to make appropriate conformational changes necessary for agonist binding. Published studies on the LAPS, in which receptor proteins were immobilized using antibodies as spacer molecules, have shown greater agonist binding affinity than obtained on the fiber optic sensor¹⁰.

To test whether spacer arms increase the conformational freedom of receptor protein immobilized on the fiber optic sensor, the nAChR was tethered to the fiber using an anti-nAChR antibody. This study focused on the nAChR of the neuromuscular junction as the molecular recognition element of a biosensor since it has been most extensively characterized and is readily purified in milligram quantities from the Torpedo electric organ. The interaction between nAChR immobilized on quartz optic fibers and nicotinic receptor agonists and antagonists was measured using the principles of total internal reflection extrinsic fluorescence spectroscopy¹¹ using a fluorescent ligand to probe receptor interactions.

2. MATERIALS AND METHODS

2.1 Preparation of Biochemicals

The nAChR from Torpedo electric organ was purified as previously described⁽¹²⁾. Briefly, Triton X-100 extracts of electric organ were mixed with a Naja α -neurotoxin (NTX) linked Sepharose gel and the bound nAChR eluted with 1 M carbamylcholine. The receptor was then extensively dialyzed against 5 mM Tris buffer, pH 7.2 to remove the carbamylcholine and detergent. The purified receptor was stored frozen at -70°C until use. Monoclonal antibodies (mAb) to nAChR were produced by hybridoma mAb35 from American Type Culture Collection (Rockville, MD). This hybridoma secretes a Rat IgG1 antibody (Ab) that binds to the receptor without interfering with the binding sites. Anti-rat Ab and other biochemicals were purchased from Sigma Chemicals (St. Louis, MO). 3-aminopropyltriethoxysilane (APTES) was purchased from Pierce (Rockford, IL). The fluorescent probe, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene labeled α -bungarotoxin (BODIPY®-BGTx) was purchased from Molecular Probes (Eugene OR). BODIPY® is a yellow-green fluorescent dye with an absorbance and quantum yield slightly higher than fluorescein, its fluorescence is not pH sensitive nor is it quenched in water.

2.2 Apparatus

A fiber optic evanescent wave sensor, designed and built at ORD, Inc. (North Salem, NH) was used for all experimentation. This instrument included a 10-W Welch Allyn quartz halogen lamp, a Hamamatsu S-1087 silicon detector, an Ismatec fixed speed peristaltic pump, a strip chart recorder and band-pass filters and lenses as indicated in the schematic (Figure 1). The quartz fibers, 1 mm in diameter with polished ends, were obtained from ORD, Inc.

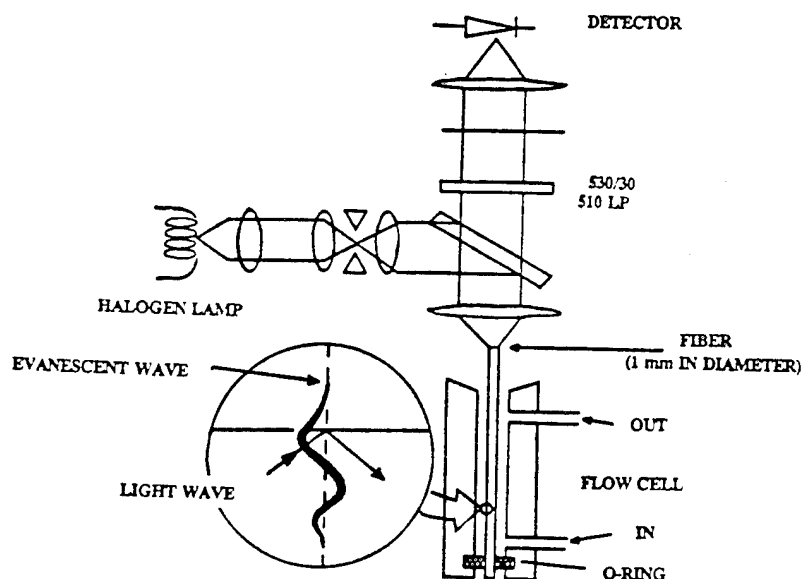


Figure 1. Schematic Presentation of Optical System Used to Measure Fluorescence.

The sensor makes use of the evanescent wave effect by exciting a fluorophore just outside the waveguide boundary (excitation wavelength = 485/20 nm). A portion of the resultant fluorophore emission is trapped in the waveguide and is transmitted back up the fiber. This emitted wavelength is detected after transmission through 510 long pass and 530/30 nm filters. The flow cell allowed the center 47 mm of a 60 mm long fiber to be immersed in 46 μ l which was exchanged every 14 sec.

2.3 Preparation of Fibers

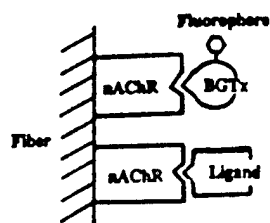
Prior to silanization, quartz fibers were acid cleaned by immersion in a concentrated hydrochloric acid/methanol (1:1) solution for 30 min, followed by several rinses in distilled water. After washing, the fibers were soaked in concentrated sulfuric acid for 30 min and rinsed in distilled water. Fibers were then boiled 30 min in distilled water to remove any remaining acid and dried on a lint free cloth. Cleaned fibers were incubated (90° C, 10 min) in 10% v/v APTES in ethanol. Following incubation, the fibers were washed with distilled water and dried overnight in a 115° C oven. Silanized fibers were then activated by incubating in glutaraldehyde (3%) for 90 min, followed by incubation in 50 μ g/ml solution of anti-rat Ab in phosphate-buffered saline (PBS) (pH 7.4) for 30 min. The anti-rat Ab loaded fibers were then incubated in rat anti-nAChR serum for 24 hrs, followed by a 4 hr incubation in purified nAChR (50 μ g/ml in citrate buffer pH 5.0). All incubations were carried out at 4° C.

2.4 Fluorescence Measurements

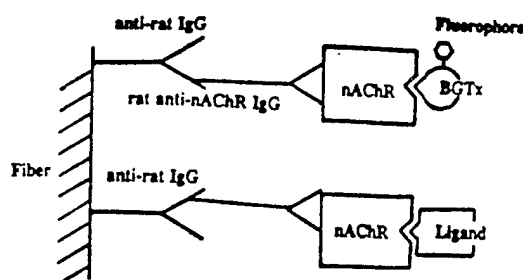
After immobilizing the receptors, the fiber was placed in the flow cell of the instrument and perfused for 5 min with PBS containing 0.5 mg/ml casein (PBS/cas) to eliminate nonspecific binding. The fiber was then perfused with 100 nM BODIPY®-BGTX in PBS/cas and competing ligands as specified in the results. Between experiments, the flow cell was washed in 1% sodium dodecyl sulfate (SDS) for 2 min followed by PBS for 10 min.

3. RESULTS

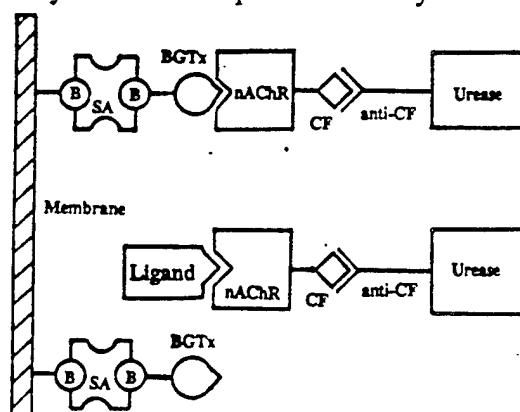
Previous fiber optic sensor studies were performed with nAChR directly immobilized onto the fiber (Figure 2a). Incubation of the antibody-coated fiber and receptor yielded a complex tethered configuration of anti-rat/anti-nAChR/nAChR (Figure 2b). The LAPS studies previously mentioned used a biotin-BGTx/carboxyfluorescein-nAChR complex (Figure 2c). The binding event of BODIPY®-BGTx to the nAChR was optically detected and plotted as millivolt/min. Initial rates were determined graphically from tracing of the millivolt response vs time, and response was measured as percent of maximum initial rate.



a. Nicotinic acetylcholine receptor non-covalently adsorbed onto quartz fiber.



b. Nicotinic acetylcholine receptor covalently tethered onto quartz fiber.



c. LAPS technology immobilization

Figure 2. Schematics of Receptor Immobilization

The addition of increasing molar concentrations of agonist to the BODIPY®-BGTx solution reduced the initial rate in a dose-dependent manner (Figure 3). Antagonists d-tubocurarine and Naja α -neurotoxin also reduced the response in a dose-dependent manner (Figure 4). The IC_{50} values (the ligand concentration that inhibited the maximum signal by 50%) of the agonists and antagonists were determined by Log-Probit analysis. K_i values were determined by the relationship $K_i = IC_{50} / (1 + [L] / K_d)$, where L is the concentration of the fluorescent tag. The K_i values for antagonists were similar to those from previous studies, while the K_i values for acetylcholine and carbamylcholine were at least one order of magnitude lower than previously reported. With the exception of Naja α -neurotoxin, the values derived from the LAPS were two orders of magnitude lower than values obtained from the fiber optic biosensor (Table 1).

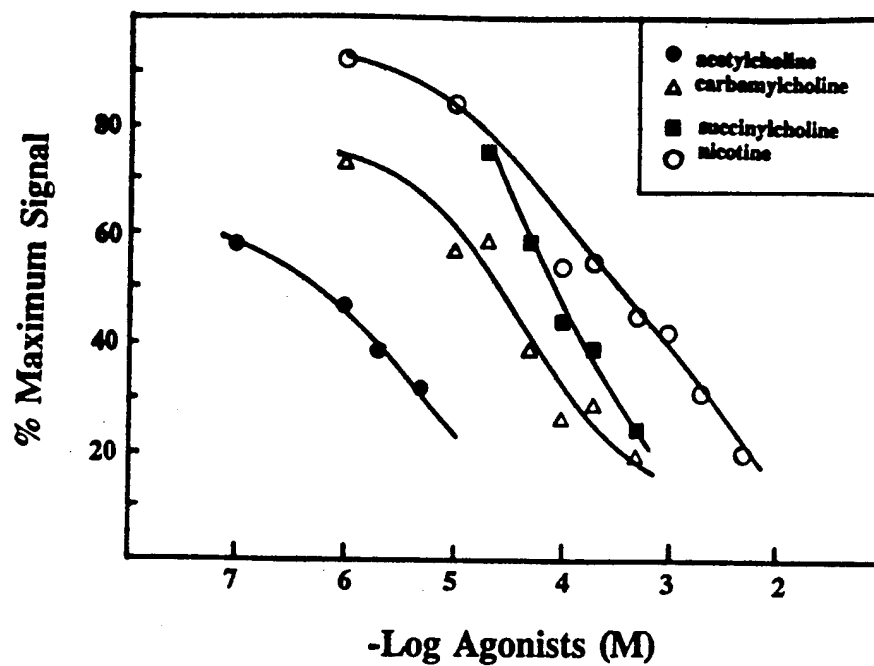


Figure 3. Competition of BODIPY-BGTx with nAChR Agonists.

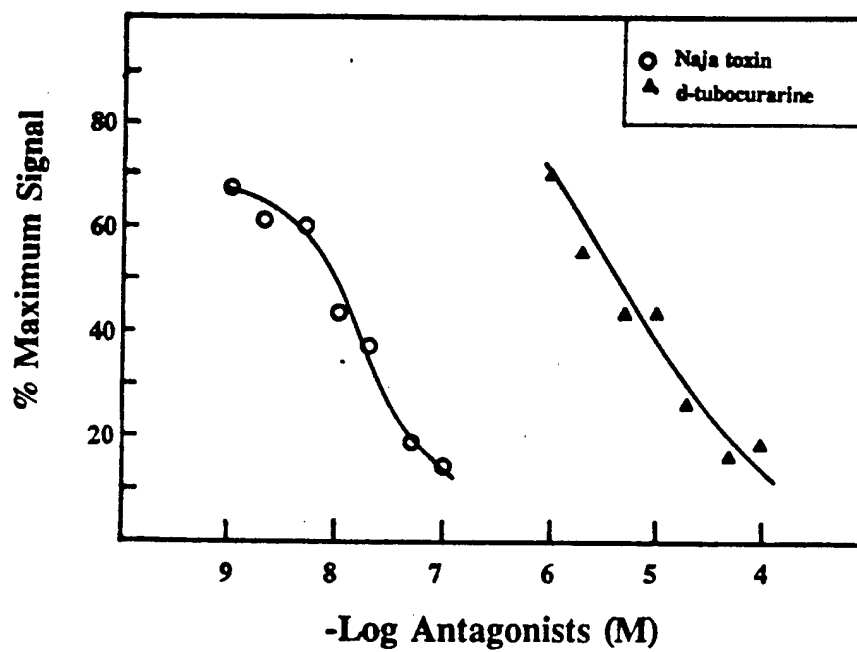


Figure 4. Competition of BODIPY-BGTx with nAChR antagonists.

Table. Fiber Optics

	Fiber-optic biosensor, tethered K_i (M)	Fiber-optic biosensor K_i (M) ¹⁰	LAPS biosensor K_i (M) ¹⁰
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Agonists			
Acetylcholine	4.7×10^{-7}	1.2×10^{-5}	2.9×10^{-8}
Carbamylcholine	7.8×10^{-5}	4.5×10^{-3}	4.6×10^{-7}
Nicotine	3.5×10^{-4}	6.0×10^{-5}	4.4×10^{-6}
Succinylcholine	2.2×10^{-5}	2.4×10^{-5}	9.0×10^{-7}
Antagonists			
δ -Tubocurarine	4.0×10^{-6}	8.8×10^{-6}	2.1×10^{-7}
α -Naja toxin	3.1×10^{-10}	1.0×10^{-8}	1.2×10^{-8}
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4. CONCLUSIONS

The optical signal generated by BODIPY®-BGTx association with nAChR tethered fibers exhibits dose-dependent inhibition by both nicotinic agonists and antagonists. While the K_i values for antagonists are similar to those obtained in membrane binding assays, the agonists appear less potent in competing with the neurotoxins in the fiber optic sensor than in membrane assays; however, the nAChR tethered onto quartz fibers binds agonists with greater sensitivity than nAChR directly adsorbed onto the fiber. This suggests that conformational freedom in the receptor complex is increased in the tethered state.

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